The Buoyant Behavior of Viral and Bacterial DNA in Alkaline CsCl

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*THE BUOYANT BEHAVIOR OF VIRAL AND BACTERIAL DNA IN ALKALINE CsCl**, †

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In equilibrium density gradient centrifugation, the banding polymer species is electrically neutral. The banding species for a negative polyelectrolyte with a polyanion P_n^{-zn} (where n is the degree of polymerization, and z the titration charge per monomer unit) in a CsCl salt gradient is $Cs_{zn}P_n$.[1] If the ion P_n^{-zn} is itself a weak acid, it may be titrated to the state $P_n^{-(zn+y)}$ by CsOH; the banding species is then $Cs_{(zn+y)}P_n$. Because of the large mass and high effective "density" of a Cs^+ ion, it is to be expected that the buoyant density in a CsCl gradient of a polymer acid will be increased by such a partial alkaline titration with CsOH. This expectation has been confirmed for polyglutamic acid (where $z = 0$ at low pH).[2] The guanine and thymine monomer units of DNA are weak acids. The present communication is concerned with the increase in buoyant density of DNA in alkaline CsCl solutions. It is well known that the guanine and thymine protons are more readily titrated in denatured DNA than in native DNA.[3] We find that the buoyant density of denatured DNA and of single strand φX-174 DNA gradually increases as the pH of the solution is increased beyond pH 9.8. The density of native DNA is not affected until a critical pH > 11 is reached, where the DNA abruptly denatures and increases in density. Similar increases in buoyant density have been observed independently by Baldwin and Shooter in their studies of 5$\overline{\text{BU}}$-substituted DNA's in alkaline solutions.[4]

Materials and Methods.—Materials: The *E. coli* DNA prepared by the method of Marmur[5] was a gift from Dr. P. O. P. Ts'o. The *M. lysodeikticus* DNA prepared by the method of Kirby[6] was a gift from Dr. T. W. Thompson. The T-4 DNA was prepared from T-4 $r+$ phage by the method of Mandel and Hershey.[7] Harshaw Chemical Company optical grade CsCl was used without further purification. Glass redistilled water was used.

Preparation of denatured DNA: The complementary DNA's were heat denatured by heating 40 γ/ml samples in 0.001 *M* tris buffer at pH 8 to 100°C for 10 min. The small containers were quickly chilled in ice and the contents poured onto solid CsCl. Samples of *E. coli* DNA were also alkali denatured at pH 12 at 100 γ/ml just prior to addition at 2γ/ml to buffered CsCl solution. The buoyant densities of alkali denatured material at pH 8 and pH 10.8 were the same as those found for the heat denatured material.

Buffer solutions: Solutions of 0.4 *M* reagent grade sodium carbonate or dibasic potassium phosphate were titrated with CO_2 gas or 50 per cent potassium hydroxide respectively to prepare carbonate buffers at pH 9.50 and 10.00 and phosphate buffers at pH 10.78, 11.18, 11.73, and 12.45. These solutions were stored in tightly closed polyethylene bottles and were checked frequently for pH.

Preparation of the alkaline DNA-cesium chloride solutions: The solutions for ultracentrifuge runs were prepared by volumetric additions of the following solutions to a 5 ml vial: CsCl, ρ = 1.900 gm/ml, 0.2 ml buffer, H_2O, and DNA solution to a final volume of 2.00 ml. The refractive index was checked, water or CsCl solution added if necessary, and 0.70 ml filled into the centrifuge cell. Without delay the remainder of the solution was poured into a 5 ml beaker and the pH measured with a small general purpose Beckman glass electrode, which had just been standardized against pH 10.0 buffer. For example, in the preparation of solutions for the φX-174 DNA buoyant

density study the following pH values were measured in the final solutions containing the above buffers at 0.04 *M:* 10.00, 10.50, 11.05, 11.50, 11.88, and 12.5. It is recognized that a potassium correction may be necessary for the more alkaline buffers. The pH values reported here are therefore regarded as reproducible instrument readings. It should be noted that the high concentration of CsCl is not likely to increase the junction potential because of the close similarity of the mobilities of the cesium and chloride ions.

Analytical ultracentrifuge: All runs were performed in Kel-F centerpieces in a Model E Spinco analytical ultracentrifuge at 25°. Two cells were run simultaneously and ultraviolet photographs of each cell were made with an alternating aperture mask. One cell of the pair was assembled with a lateral −1° bottom window. Both cells were assembled with longitudinal −1° windows. All runs were for at least 24 hr.

Measurement of buoyant density: The densities of the alkaline solutions were measured pycnometrically in 300 microliter pipets. Refractometry was avoided because of the complications caused by buffer addition. The buoyant density, i.e., the density of the solution in which the band forms at the root mean square position in a *ca.* 1.2 cm liquid column, was carefully determined for native T-4 DNA in alkaline solution between pH 8 and 10.8 and for denatured T-4 DNA between pH 11.5 and pH 12.5 by the procedure described by Vinograd and Hearst.[8] The T-4 DNA was used as a marker in the study of the other DNA's. In several twin-cell experiments the buoyant densities obtained in solutions buffered with cesium carbonate were compared with those in sodium or potassium carbonate solutions. The lighter cations caused a reduction of 0.006 gm/ml in the buoyant density. All of the measured buoyant densities in buffered alkaline solutions have been corrected by addition of 0.006 gm/ml.

Results.—The essential results are displayed in Figure 1. The buoyant densities of the native DNA's are substantially constant up to the pH at which denaturation occurs, and the density abruptly increases. The buoyant densities of denatured DNA's and of φX-174 DNA increase by about 0.045 units over about one unit of pH. This increase results from the titration of guanine and thymine residues.

Titration curves for single strand DNA's: The titration curve of a monomer acid has a width of two pH units between the 10 and 90 per cent titration points. The titration curve of a typical polyelectrolyte acid at moderate salt concentrations is usually several pH units broader than the titration curve of the monomer and the midpoint is displaced somewhat to higher pH, because of electrostatic repulsion effects.

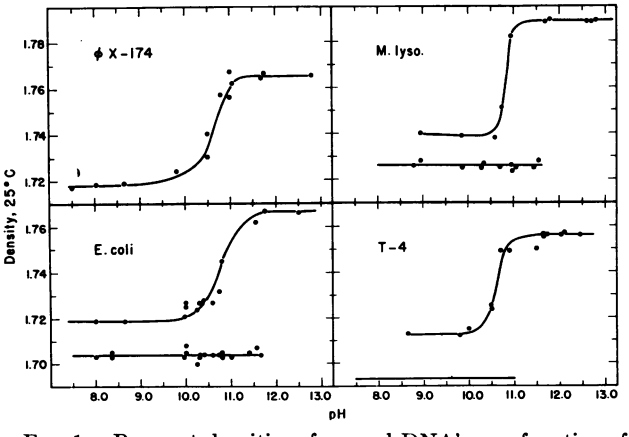

FIG. 1.—Buoyant densities of several DNA's as a function of pH at 25°. In each case the lower set of points is for the native material. The data for native T-4 DNA are not shown. Twenty-one concordant points were obtained with a standard deviation of ±0.0026.

The midpoints and 10–90 per cent widths of the density titration curves for φX-174 DNA and the heat denatured DNA's are: φX DNA, 10.6, 1.1; T-4 phage DNA, 10.6, 0.6; *M. lysodeikticus* DNA, 10.8, 0.4; *E. coli* DNA, 10.7, 1.1. The pK's of guanylic and thymidylic acids in dilute aqueous solution are reported as 9.7 and 10.0;[9] the pK of 5′ guanylic acid in 6.4 *M* CsCl is 9.9,[10] and we assume that CsCl has a similar small effect on thymidylic acid. The buoyant density change is probably approximately proportional to the degree of titration. Thus the titration curves for the denatured DNA's are displaced to higher pH by about one unit compared to the monomer but are narrower. The combination of displacement and sharpening shows that there are cooperative effects due to a substantial amount of base pairing in the denatured state before titration. Electrostatic shielding effects in the concentrated CsCl solution may also contribute to the lack of broadness in the titration curve.

The alkaline titration curves of denatured DNA at moderate ionic strengths (e.g., 0.10 *M*) also seem to be relatively narrow and displaced to high pH because of residual base pairing.[3] The optical properties of denatured DNA also indicate residual secondary structure.[11]

Density changes, secondary structure, and hydration: The buoyant densities are listed in Table 1. We observe the following relations for the complementary DNA's. The change in buoyant density in going from the native material to the fully titrated denatured form at alkaline pH is constant (*N*–*L* in Table 1). The

TABLE 1

BUOYANT DENSITIES OF NATIVE AND DENATURED DNA'S AS A FUNCTION OF pH

DNA	Mole fraction *GC*	Native pH 7–9 *L*	Denatured pH 7–9 *M*	Denatured alkaline *N*	Density shifts *M*–*L*	Density shifts *N*–*M*	Density shifts *N*–*L*
T-4	0.34	1.693	1.712_5	1.756	0.019_5	0.043_5	0.063
E. coli	0.50	1.704	1.719	1.766	0.015	0.047	0.062
M. lysodeikticus	0.72	1.726	1.739	1.788_5	0.013	0.049_5	0.062_5
φX-174	...	...	1.718	1.765	...	0.047	...

buoyant density change on denaturation at neutral pH (*M*–*L*) decreases with increasing GC content. Correspondingly, the density change on alkaline titration of the denatured DNA's (*N*–*M*) increases with increasing GC content. These trends indicate that the amount of secondary structure in the denatured DNA's at pH 7 increases with GC content. The fact that there is no dependence on base composition for the total density change from the neutral to the fully titrated material shows that the amount of residual secondary structure in the fully titrated DNA's is independent of base composition and is probably zero.

The average G + T content of the denatured DNA's derived from a complementary two-stranded DNA is 0.50 mole fraction, but the G + T content of φX DNA is 0.57.[12] From this factor alone, the density change for complete alkaline titration of φX DNA would be predicted to be 14 per cent greater than for a complementary DNA. The observed density change is about the same as for the complementary DNA's. The discrepancy may be due to a smaller amount of secondary structure in φX DNA, or it may be due to other structural effects, depending on base composition, which are not understood. (The optical data suggest that φX DNA is not deficient in secondary structure as compared to denatured DNA.[12])

The buoyant density change of a denatured DNA for complete alkaline titration

is less than expected for replacement of a covalently bound hydrogen atom by an anhydrous cesium ion. The following calculation indicates that the net hydration of the DNA increases upon alkaline titration. Consider T-4 DNA, for which the amount of secondary structure in the denatured form is the least of the three complementary DNA's studied. Take 2.12 as the buoyant density of anhydrous T-4 DNA;[13] and 944 as the formula weight of a Cs_2B_2 base pair (where B is a nucleotide monomer). Assume that the actual buoyant density of 1.712 in 7 *M* CsCl implies hydration by water with a density of 1.00. One then calculates that there are 14 molecules of water of hydration per base pair. The fully titrated alkaline DNA has a formula of Cs_3B_2; the formula weight per base pair is 1076. Assume a difference of molar volume for the anhydrous Cs^+ ion and displaced hydrogen atom of 32 cc.[13] The density of 1.756 in alkali corresponds to a hydration of 17 molecules of water per base pair. Such an increase in hydration of about 3 molecules of water per additional charge seems reasonable.

Band widths and the problem of complement separation: For a two-stranded Watson-Crick base-paired DNA, $X_A = X_T$ and $X_G = X_C$, so that $X_T + X_G$ is 0.50 mole fraction (where X_A is the mole fraction of adenine, etc.). There is no requirement however that $X_T + X_G$ in one strand should equal $X_T + X_G$ in the other. Thus, density gradient centrifugation in alkaline solution offers the possibility of separation of complementary strands. The fact is however that we observed no bimodal density distributions for the complementary DNA's in alkaline solution. In general, broader bands were observed for the fully titrated denatured materials at alkaline pH's than for the native DNA's at pH 7. This broadening might in part be due to the separation of complementary strands but it could also be due to a pronounced decrease in molecular weight and/or an increase in density heterogeneity resulting from single strand breaks present in the initial material. (We have ourselves obtained no evidence that the two strands of a complementary DNA do come apart on alkaline denaturation. However it has been reported that upon denaturation at pH 12 of heavy isotope hybrid *E. coli* DNA followed by density gradient centrifugation at pH 7, two bands are formed.[14] This shows that the two strands do come apart.) The possible separation of complementary strands from the DNA's of certain viruses by density gradient centrifugation of denatured materials at neutral pH has recently been reported.[15]

In order to minimize the amount of breakage during preparation, an experiment was done in which T-4 DNA was prepared by adding the bacteriophage directly to the centrifuge cell and then adding CsCl at pH 11.5. The optical density in the band indicated substantially complete lysis. The buoyant density was as expected, and the band profile corresponded now to an anhydrous molecular weight for Cs DNA of thirty million, approximately half the value obtained in comparable experiments at neutral pH with phenol extracted T-4 DNA. Thus again there is no clear evidence for complement separation.

Two experiments were performed with protoplasts from *E. coli* K12, which were lysed directly in the centrifuge cell by adding 0.010 ml of water at 5°C to 0.002 ml of a suspension containing 10^8 protoplasts. The addition of alkaline CsCl, pH 11.5, and subsequent centrifugation first at 10° and then at 25°, gave two native DNA bands at both temperatures, with densities close to 1.71 and differing by about 0.002. Each of these bands was approximately $^1/_5$ as wide as is normal for *E. coli* DNA

prepared by the variety of procedures discussed by Marmur.[5] In these experiments the alkaline hydrolysis of the sedimented RNA was signalized by the gradual invasion of the bottom fourth of the cell by optically absorbing material. It is apparent that the DNA in this organism under conditions in which deproteination is extensive is in a higher molecular weight form than has previously been observed in banding experiments. This result is consistent with the autoradiographic findings of very long DNA obtained by gentle lysis.[16] At the present time the significance of the duality of the *E. coli* DNA is not understood. These incomplete experiments are mentioned merely to suggest that alkaline CsCl may be a useful reagent for the extraction of DNA from viruses and bacteria with a minimum amount of degradation.

An important question for this study is the stability of DNA in alkaline solution. Some preliminary experiments bearing on this point have been made. A series of solutions containing 1 γ/ml of φX DNA were prepared in CsCl at varying pH and a final density of 1.70 gm/ml. These solutions were stored at 25°C for 48 hr and then assayed by the *E. coli* K-12 protoplast procedure of Guthrie and Sinsheimer.[17] The infectivities of the samples expressed as per cent survivors and normalized with the result obtained at pH 8.5 were 60, 67, 25, 44, and 25 at pH's 10.0, 10.5, 11.5, 12.0, and 12.5 respectively. These results indicate an average of about one lethal hit per φX-174 molecule in 48 hr at pH 11 (MW = 1.7×10^6). However it is clear from the sharpness of the T-4 bands in the fully titrated single strand material referred to above that there is not more than one or two physical breaks per molecule of 6×10^7 molecular weight in a typical 64-hr centrifuge experiment at alkaline pH. It has been reported that in 0.20 *M* NaOH–1% CH_2O at 25°, denatured T-4 DNA suffers one chain break per molecule per 40 hr.[18]

Denaturation and renaturation: Renaturation was observed with heat or alkali denatured T-4 DNA at pH's between 10.5 and 10.9. With amounts of DNA of the order of 40 μg/ml at band center, bands formed at the expected position for denatured DNA and then moved in the course of 20 hr and fused with the native band present as a marker. This renaturation was not observed when the concentration was reduced by a factor of 5 to 10. Thus, the renaturation is concentration dependent. At pH 7 at room temperature, quenched, denatured T-4 DNA was not observed to renature in the centrifuge. This is presumably due to the stability of the randomly formed hydrogen bonds. At pH 10.5 to 10.9, there is considerable dissociation of the short ordered regions and the conditions for reannealing are more favorable.

A few experiments were performed to observe the melting of native T-4 DNA at pH 10.8. After formation of the native band in the centrifuge at 25°, the temperature was gradually raised. In one experiment a melting point of 31° was found and the expected band shift of about 0.053 density units was seen. In a second experiment the melting point was apparently over 40°. These results show that melting studies can be performed at sedimentation equilibrium in a density gradient, but require very close pH control.

Summary.—Denatured DNA increases in buoyant density by about 0.045 density units in alkaline CsCl solution because of the titration of the N–H protons of the thymine and guanine residues. The pH at the midpoint and the narrow width of the titration curve indicate considerable residual hydrogen bonding in the denatured

state at neutral pH. The density of native DNA is not affected until a critical pH > 11 is reached, where the DNA abruptly denatures and increases in density. The change in buoyant density in going from the native material at neutral pH to the fully titrated denatured form at alkaline pH is constant and independent of the base composition. Density gradient centrifugation in alkaline CsCl has been demonstrated to be a useful method for the separation of native and denatured DNA. It may also be useful for the separation of complementary strands.

A neutral formaldehyde solution has been recommended as a medium for the study of single stranded DNA's obtained by denaturation without complications due to reannealing.[19] Alkaline solutions (without formaldehyde) are versatile in that at a pH somewhat below the critical pH for denaturation they are good reannealing media; at a higher pH, they are probably suitable for the study of independent separated strands.

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[1] The buoyant density of DNA decreases when LiBr is added to a DNA solution in CsBr because of preferential binding of the Li^+ by the DNA (Hearst, J. E., and J. Vinograd, these PROCEEDINGS, **47**, 825 (1961)); in pure CsBr however, the buoyant species must be the CsDNA salt, irrespective of the amount of ion association between the polyanion and the Cs^+ ions.

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